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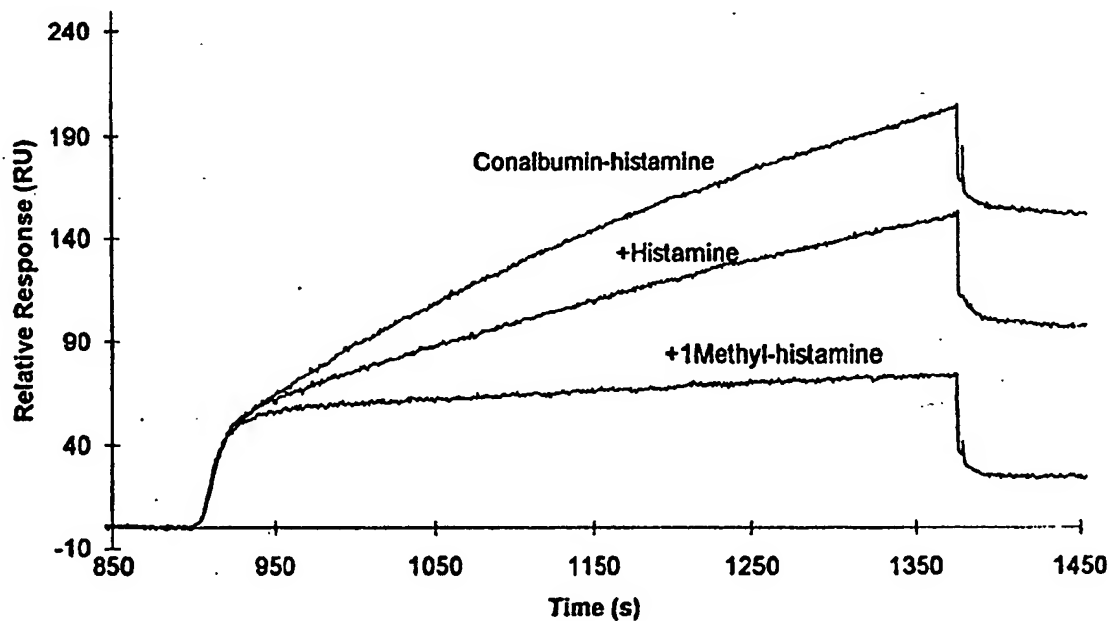
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: A METHOD OF DETERMINING AFFINITY AND KINETIC PROPERTIES

(57) Abstract

A method of determining the affinity and kinetic properties of low molecular weight ligands for their interaction with a common receptor comprises the steps of immobilizing the receptor on a sensing structure, mixing in known proportions each ligand with a ligand analogue the response of which on the sensing structure is substantially higher than that of the low molecular weight ligands, contacting each mixture with the sensing structure and measuring the response, and comparing the response of each mixture with that of the ligand analogue alone, the distortion of the ligand analogue response being representative of the affinity and kinetic properties of the ligand.

A METHOD OF DETERMINING AFFINITY AND KINETIC PROPERTIES

The present invention relates to the determination of
the affinity and kinetic properties of particularly low
5   molecular weight compounds with regard to their
interactions with a receptor molecule.

Ligand-receptor interactions are usually characterized
in the terms of the binding affinity. Determination of rate
constants would be helpful to characterize the interaction,
10   but unfortunately only a few methods are available where
the kinetic properties of the interaction can be directly
visualized. One such method, which has recently been
described (Karlsson R. et al., J. Immunol. Meth. 145, 229-
240, 1991), uses mass sensitive detection and a flow system
15   for direct kinetic analysis of ligands interacting with
their immobilized receptors at a sensor surface. However,
this method is restricted to the study of high molecular
weight ligands, the increase of mass on the sensor surface
in the case of low molecular weight components (M_W <
20   approx. 3000 Da) which interact with an immobilized
receptor being too small to be accurately measured.

EP-A-276 142 discloses a method of quantitatively
measuring, on a surface of an optical structure capable of
exhibiting surface plasmon resonance (SPR), small
25   molecules, such as drugs and hormones, using a competitive
assay format. The method is characterized in that the
sample antigen will compete with the same antigen (or an
antigen analogue) coupled to a comparatively large (optical
thickness enhancing) "label particle", such as a latex
30   particle, for the binding to an immobilized receptor. The
amount of labelled antigen bound will then be inversely
proportional to the concentration of antigen in the sample.

Rogers, K. R., et al., Biosensors & Bioelectronics 6
(1991) 507-516 describes the use of fluorescence-labelled
35   ligands to determine the number of free sites on a receptor
bound to a fibre-optical biosensor. To determine the
affinity for the reaction of a non-labelled ligand with the
receptor, the receptor is incubated with non-labelled

ligand, a fluorescence-labelled ligand is added and the
fluorescence measured, and the measured fluorescence signal
is compared with that obtained when the receptor is reacted
directly with the fluorescence-labelled ligand. When the
5   procedure is repeated with a number of concentrations of
non-labeled ligand, the affinity constant for the
interaction between the non-labeled ligand and the receptor
may be calculated.

In accordance with the present invention, it has now
10   been found that the lack of sensitivity of mass-sensing
methods to accurately detect low molecular molecules when
the size of the receptor molecule is much larger than the
molecule to be detected may be advantageously utilized to
compare by competitive kinetics the affinity and kinetic
15   properties of different low molecular weight components
interacting with the same receptor, using an approach
similar to that used for the concentration measurements in
EP-A-276 142 above.

According to this novel concept of competitive
20   kinetics, two ligands are allowed to react simultaneously
with the binding site of a common immobilised receptor. One
of the ligands is of high molecular weight and the other is
one of low molecular weight. The progress of the binding
interaction with the receptor is studied by recording the
25   binding curves (plotting of the detector response versus
time) for, on one hand, each ligand alone and, on the other
hand, when the high molecular weight ligand and the low
molecular weight ligand compete for the binding site of the
receptor. In the latter case, both ligands bind to the
30   receptor but almost the entire signal will be due to the
binding of the high molecular weight component. The binding
of the low molecular weight component is instead seen as a
distortion of the binding curve obtained with the high
molecular weight ligand alone. The change in shape and in
35   position of the binding curve obtained under competing
conditions thus reflects the kinetic and affinity
properties of the low molecular weight ligand-receptor
interaction.

By proceeding in this way, for example, low molecular weight ligands may be affinity ranked relative to each other by studying their influence on the binding of a high molecular ligand analogue to the same receptor. This may be

5   favourably applied in _inter alia_ drug screening.

The present invention therefore generally relates to a novel method of determining at least the relative affinities and kinetic properties of low molecular weight ligands for their interactions with a common receptor,

10   which method is characterized by immobilizing the receptor on a sensing structure, mixing in known proportions each low molecular weight ligand with a ligand analogue the response of which on the sensing structure is substantially higher than that of the low molecular weight ligands,

15   contacting each mixture with the sensing structure and measuring the response, and comparing the response of each mixture with that of the ligand analogue alone, the distortion of the ligand analogue response being representative of the affinity and kinetic properties of

20   the ligand.

By the expression "substantially higher", with regard to the ligand analogue response is to be understood that the contribution of the ligand analogue response in a mixture with a low molecular weight ligand should represent

25   the major part of the response of the mixture. Preferably, the response of the ligand analogue is from about 20 times that of low molecular weight ligand (i.e. about 95%) to, say, about 100 times that of low molecular weight ligand. Such relatively higher response of the ligand analogue may

30   be inherent in the basic molecule per se or may be provided by a species bound to the basic ligand analogue molecule.

An advantageous feature of the invention is that it is not necessary to study the binding curve up to steady state, or equilibrium, as is the case in the concentration

35   measurement proposed in the above EP-A-276 142, but the initial part of the binding curve is sufficient. It is readily seen that a considerable amount of time will be saved thereby.

Preferably, however, also the dissociation phase is studied, i.e. the dissociation of the bound ligands from the sensor surface when the surface is no longer contacted with the ligand solution.

5   The low molecular weight ligand may be a drug or a potential drug reacting with its receptor, or it may be the binding part obtained from a larger molecule interacting with the binding partner of the parent molecule.

In a preferred embodiment, the ligand analogue is a 10 high molecular weight molecule.

Such high molecular weight ligand analogue may be an antibody or the binding part of an antibody, but preferably the high molecular weight ligand is a conjugate of one of the low molecular ligands and a high molecular weight 15 component. "High molecular weight", which term is to be interpreted broadly, may for the present purposes be from, say, about 5000 Dalton to about 1000 x the molecular weight of the low molecular weight component. In the case where the low molecular weight ligand is obtained from a larger 20 molecule (the low molecular weight component being, for example, a peptide or an oligonucleotide), the high molecular weight ligand may be the parent molecule.

Preferably, the high molecular weight ligand reacts in a one-to-one fashion with the immobilised binding partner, 25 the interaction preferably being characterized by a high dissociation rate constant ($0.001 < k_{diss} < 0.1$).

The low molecular weight ligands and the ligand analogue are preferably introduced over the sensor surface in such a way that for each component the concentration may 30 be regarded as constant over time, and thereby pseudo first order rection kinetics may be assumed.

While a stationary assay format may be used, it is preferred to use a dynamic format, the low molecular weight ligands and the ligand analogues, respectively, being 35 introduced over the sensor surface in a fluid flow.

For screening purposes, the low molecular molecular weight ligands and the ligand analogues may be presented simultaneously onto an array of sensor surfaces (such

arrays of sensor surfaces being known per se in the art)
with either different receptors or different amounts of a
single receptor immobilised.

The invention is generally applicable to those
5   detection methods where the parameter being measured is
generated from both the ligand and the ligand analogue,
such as e.g. mass, charge, dielectric constant etc. Among
these methods are, for example, mass detecting methods,
such as piezoelectric, optical and thermo-optical methods,
10   and electrochemical methods, such as potentiometric,
conductometric and amperometric methods.

Among optical methods may particularly be mentioned
those that detect mass surface concentration, such as
reflection-optical methods, including both internal and
15   external reflection methods, e.g. ellipsometry and
evanescent wave spectroscopy (EWS), the latter including
surface plasmon resonance spectroscopy (SPRS), Brewster
angle reflectometry, critical angle reflectometry,
frustrated total reflection (FTR), evanescent wave
20   ellipsometry, scattered total internal reflection (STIR),
optical waveguide sensors, etc.

SPR-based detection methods have attracted much
attention lately. The phenomenon of SPR is well known. In
brief, SPR is observed as a dip in intensity of light
25   reflected at a specific angle from the interface between an
optically transparent material, e.g. glass, and a thin
metal film, usually silver or gold, and depends on among
other factors the refractive index of the medium (e.g. a
sample solution) close to the metal surface. A change of
30   refractive index at the metal surface, such as by the
adsorption or binding of material thereto, will cause a
corresponding shift in the angle at which SPR occurs. To
couple the light to the interface such that SPR arises, two
alternative arrangements are used, either a metallized
35   diffraction grating (Wood's effect), or a metallized glass
prism or a prism in optical contact with a metallized glass
substrate (Kretschmann effect). For further details on SPR,
reference is made to our WO 90/05295. In an SPR-based

assay, a receptor is bound to the metal surface, and the
interaction thereof with a ligand in a solution in contact
with the surface is monitored.

    A commercial SPR-based biosensor system based on the
5   Kretschmann effect (BIAcore^{TM} system, supplied by Pharmacia
Biosensor AB, Uppsala, Sweden) was used in the following
non-limiting examples which illustrate the method of the
invention.

<h3 style="text-align:center">EXAMPLE 1</h3>

10   <h3 style="text-align:center">Preparation of sensor surfaces</h3>

    A continuous flow of HBS (10 mM Hepes with 0.15 M
NaCl, 3.4 mM EDTA and 0.05% surfactant P20 at pH 7.4) was
maintained over a BIAcore^{TM} sensor chip CM5 (a gold-plated
glass slide supporting a carboxymethyldextran layer via a
15   monolayer of long-chain hydrocarbons; supplied by Pharmacia
Biosensor AB, Uppsala, Sweden). The carboxylated matrix on
the sensor surface was activated by a three-minute
injection of a solution containing 0.2 M N-ethyl-N'-(3-
diethylaminopropyl)carbodiimide (EDC) and 0.05 M N-
20   hydroxysuccinimide (NHS). 100 μg/ml of rabbit anti-mouse Fc
(RAMF$_c$) antibodies (Pharmacia Biosensor AB, Uppsala,
Sweden) in 10 mM acetate buffer, pH 4.75, were then
injected followed by a pulse of ethanolamine-hydrochloride,
pH 8.5, to block remaining NHS-ester groups.

25   <h3 style="text-align:center">Binding of anti-p24-Mab to the sensor surface and kinetic
analysis of p24 ligand interaction</h3>

    With the above prepared sensor surface docked into the
BIAcore^{TM} instrument, a solution of purified monoclonal
anti-p24 antibody (Pharmacia Diagnostics AB, Uppsala,
30   Sweden), hereinafter referred to as Mab 7, was injected,
the anti-p24 monoclonal antibody thereby being captured by
the immobilized RAMFc. Different solutions of HIV core
protein p24 (Pharmacia Genetic Engineering, San Diego,
USA), varying in concentration from 18.75 to 300 nM, were
35   then injected and the binding curves, relative response in
resonance units (RU) versus time in seconds, were recorded
(1 RU corresponds to a mass change, caused by the binding
of a protein, on the sensor surface of about 1 pg/mm^2;

Stenberg, E., et al., J. Colloid and Interface Sci. 143
(1991) 513-526).

Kinetic analysis of the Mab 7-p24 interaction revealed
that the system was extremely stable with a dissociation
rate constant less than 10^{-5} (s^{-1}) and that the association
rate constant was $2.2 \cdot 10^5$ ($M^{-1}s^{-1}$). In order to obtain a
reasonable response level for the following competitive
experiment, a p24 concentration of 60 nM was chosen. The
binding curve for 60 nM concentration is shown in Fig. 1.

Affinity of synthetic p24 derived peptides for anti-p24 Mab

100 μl volumes of a 60 nM solution of p24 in HBS were
mixed with 100 μl of different concentrations (5 nM to 1
mM) of two synthetic p24 derived peptides corresponding to
(i) the regions 329-352 (peptide 7):
DCKTILKALGPAATLEEMMTACQG (M_W=2494), and (ii) the regions
332-349 (peptide 5): TILKALGPAATLEEMMTA (M_W=1841) (obtained
from Dr Åke Engström, Department of Immunology, Biomedical
Center, University of Uppsala, Sweden). The peptide
sequences are according to Ratner et al., Nature 313, 227-
284, 1985 and are numbered according to Myers G. et al.,
Database Human Retroviruses and AIDS, Los Alamos National
Laboratory, Los Alamos, U.S.A., 1988. Each mixture was
injected at 10 μl/min for two minutes over the sensor
surface with captured anti-p24 antibody and the respective
binding curves for each peptide were recorded. Peptide 5
was used at the concentrations: 0, 4.91, 14.75, 44.26,
132.78, 398.33, and 1195.00 μM; and peptide 7 at the
concentrations 0, 0.005, 0.016, 0.049, 0.147, 0.441, and
1.323 μM.

The binding curves for peptide 5 are shown in overlay
plot in Fig. 2, and a corresponding overlay plot of the
binding curves for peptide 7 is shown in Fig. 3. Fig. 3
demonstrates that for peptide 7, competing kinetics is seen
already at 5 nM concentration, whereas for peptide 5
competition is evident only at μM concentrations, as is
seen in Fig. 2. Evidently, these two peptides have quite
different affinities for the immobilized antibody. To
demonstrate this more directly, p24/peptide mixtures with

the two peptides at equal concentrations were injected and
"the binding curves recorded (p24 at 60 nM and the peptides
at 44 μM). The results are shown in Fig. 4 which clearly
demonstrates that peptide 7 has a high affinity for the
5   antibody, whereas peptide 5 has a low affinity for the
antibody.

EXAMPLE 2

Preparation of conalbumin-aminotheophylline conjugate

30.8 mg of conalbumin (Sigma) were dissolved in 0.5 ml
10 PBS-buffer, pH 7.5. 44 μl of a 3.12 mg/ml solution of SPDP
(Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in
ethanol were added to the protein solution and allowed to
react at room temperature for one hour. SPDP-modified
conalbumin was transferred into 0.1 M acetate buffer, pH
15 4.5, by buffer exchange on a NAP5-column (Pharmacia LKB
Biotechnology AB, Uppsala, Sweden).

6.7 mg of the SPDP-modified conalbumin was reduced
with DTE, excess DTE was removed by desalting on a NAP5
column, and the SH-conalbumin preparation was again reacted
20 with SPDP but now at pH 4.5. Excess reagents were removed
by desalting on a NAP5 column and 100 μl of a 20 mM
aminotheophylline solution was added and allowed to react
with the succinimide ester of conalbumin. Excess reagents
were again removed by buffer exchange on a NAP column and
25 the conjugate was taken up in 10 mM HBS buffer. The
conjugate formed was purified by gel filtration on a
Superose™ 12 column (Pharmacia LKB Biotechnology AB,
Uppsala, Sweden). A high molecular weight fraction was
removed, and the aminotheophylline-conalbumin conjugate was
30 identified by its reaction with immobilised
aminotheophylline monoclonal antibody (Kabi Pharmacia AB,
Uppsala, Sweden) as detected in the BIAcore™ system. The
final yield was 0.15 mg.

Binding of anti-theophylline Mab to the sensor surface and
35 #### kinetic analysis of conalbumin-aminotheophylline ligand
interaction

A solution of purified monoclonal anti-theophylline
antibody (Kabi Pharmacia AB, Uppsala, Sweden) was injected

into the BIAcore^{TM} instrument as in Example 1, the anti-
theophylline antibody thereby being captured by the
immobilised RAMFc. Different solutions of the above
prepared conalbumin conjugate varying in concentration from
5   100 to 1000 nM, were then injected and the binding curves
were recorded. Kinetic analysis revealed that the
association rate constant was $3.0 \cdot 10^4$ $M^{-1}s^{-1}$ and the
dissociation rate constant was $2.2 \cdot 10^{-2}$ s^{-1}. For the
following competitive experiment, a concentration of 620 nM
10   was chosen, this concentration being close to the K_D
(k_{ass}/k_{diss}) concentration (730 nM).

Affinity of theophylline derivatives for immobilised anti-theophylline monoclonal antibody

100 μl volumes of a 620 nM solution of the conjugate
15   between aminotheophylline and conalbumin were mixed with
each of 100 μl of 500 nM solutions of theophylline (Fluka
A.G., Switzerland), 7-theophylline acetic acid (Fluka A.G.)
and aminotheophylline (Fluka A.G.), respectively. Each
mixture was injected at 10 μl/min for 90 seconds over the
20   surface with captured anti-theophylline monoclonal
antibody, and the respective binding curves were recorded.
The results are shown in Fig. 5, from which it is evident
that aminotheophylline has the highest affinity for the
antibody and that the acetic acid derivative of
25   theophylline reacts with much lower affinity. Theophylline
itself has an affinity in between.

EXAMPLE 3

Preparation of conalbumin-histamine conjugate

6.7 mg of SPDP modified conalbumin, prepared as in
30   Example 2, were reduced with DTE, excess DTE was removed by
desalting on a NAP5 column (Pharmacia LKB Biotechnology AB,
Uppsala, Sweden) against PBS buffer, and the SH-conalbumin
preparation was allowed to react overnight with a six times
molar excess of pyridyl-histamine derivative (Kabi
35   Pharmacia AB, Uppsala, Sweden). The conalbumin-histamine
conjugate was purified and tested for binding activity as
described above. The final yield of purified conjugate was
1.5 mg.

Binding of anti-histamine Mab to the sensor surface and kinetic analysis of conalbumin-histamine ligand interaction

A solution of purified monoclonal anti-histamine antibody (Kabi Pharmacia AB, Uppsala, Sweden) was injected
5   into the BIAcore™ instrument as in Example 1, the anti-histamine antibody thereby being captured by the immobilised RAMFc. Different solutions of the above prepared conalbumin conjugate, varying in concentration from 100 to 1000 nM, were then injected and the binding
10   curves were recorded. Kinetic analysis gave an association rate constant (k_{ass}) value of $2.1\cdot10^4$ $M^{-1}s^{-1}$ for the association of the histamin conjugate to the immobilised anti-histamin antibody, and a dissociation rate constant (k_{diss}) for the dissociation of the histamin conjugate from
15   the immobilised anti-histamin antibody of $4.3\cdot10^{-4}$ s^{-1}, i.e. substantially lower than for the theophylline conjugate in Example 2. For the following competitive experiment, a concentration of 80 nM was chosen.

Affinity of histamine and methylhistamine for immobilised
20   anti-histamine monoclonal antibody

100 μl volumes of a 80 nM solution of the conjugate between histamine and conalbumin were mixed with each of 100 μl of 220 nM solutions of histamine (Sigma, St. Louis. Mo., U.S.A.) and methylhistamine, respectively. Each
25   mixture was injected at 10 μl/min for 8 minutes over the surface with captured anti-histamine monoclonal antibody, and the respective binding curves were recorded. The results are shown in Fig. 6, from which it is evident that methylhistamine has a higher affinity for the antibody than
30   histamine itself.

CLAIMS

1.   A method of determining the affinity and kinetic
properties of low molecular weight ligands for their
interaction with a common receptor, **characterized** by
immobilizing the receptor on a sensing structure, mixing in
known proportions each low molecular weight ligand with a
ligand analogue the response of which on the sensing
structure is substantially higher than that of the low
molecular weight ligands, contacting each mixture with the
sensing structure and measuring the response, and comparing
the response of each mixture with that of the ligand
analogue alone, the distortion of the ligand analogue
response being representative of the affinity and kinetic
properties of the ligand.

2.   The method of claim 1, **characterized** in that the
response of the ligand analogue is at least about 20 times
that of the low molecular weight ligand.

3.   The method of claim 1 or 2, **characterized** in that the
ligand analogue is a high molecular weight molecule.

4.   The method of claim 1, 2 or 3, **characterized** by
recording a partial binding curve (response vs time) for
the ligand analogue and each mixture, respectively.

5.   The method of claim 4, **characterized** in that the
binding curve leads to equilibrium.

6.   The method of claim 4 or 5, **characterized** in that the
binding curve is an association/dissociation curve.

7.   The method of any one of claims 1 to 6, **characterized**
in that the concentration of the ligand analogue is
selected close to the K_D concentration.

8.   The method of any one of claims 1 to 7, **characterized** in that said sensing structure is a mass sensing detector.

9.   The method of claim 8, **characterized** in that said mass sensing detector is one based upon evanescent wave sensing.

10.   The method of claim 9, **characterized** in that said evanescent wave sensing is based on surface plasmon resonance.

11.   The method of any one of claims 1 to 10, **characterized** in that said low molecular ligands are potential drugs.

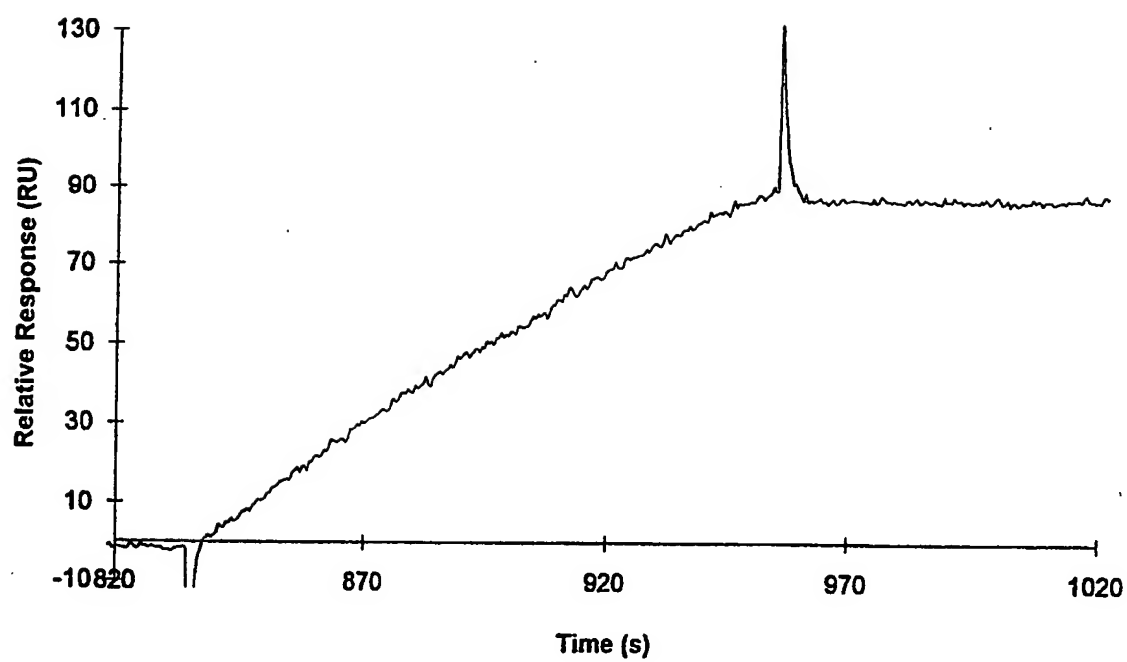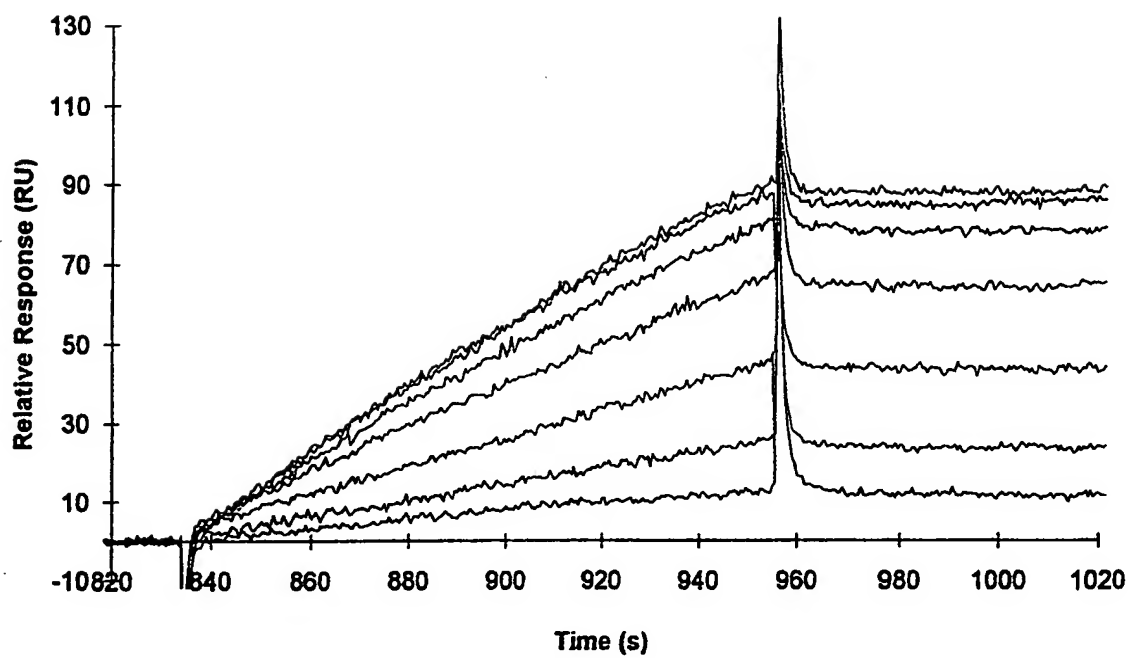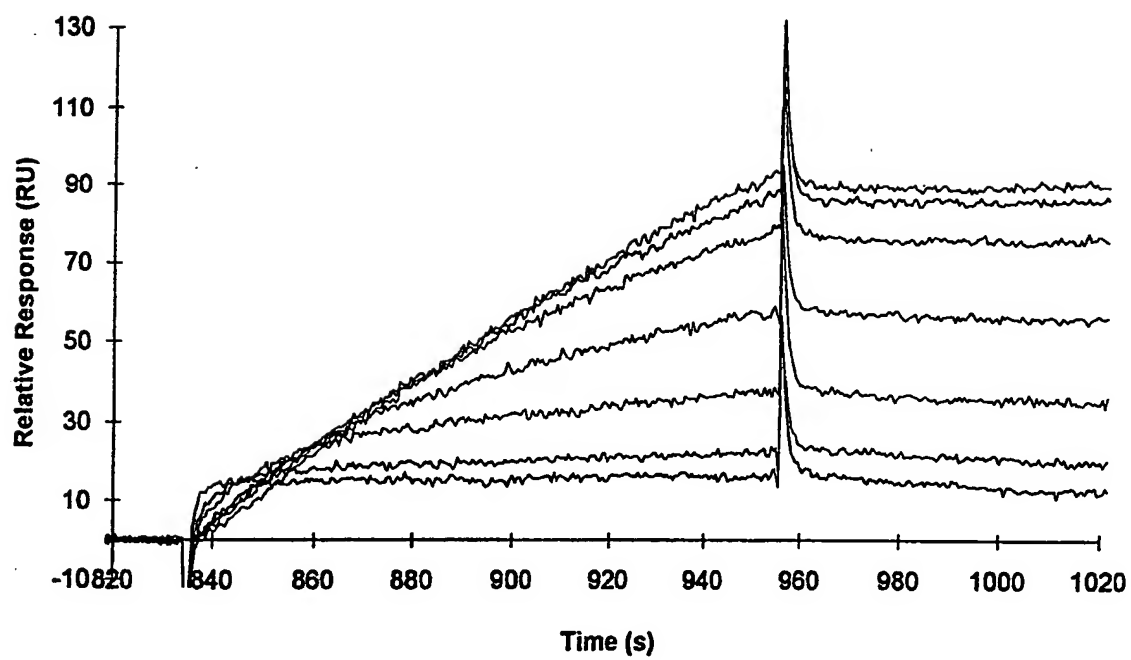FIG. 1

FIG. 2

FIG. 3

4 / 6

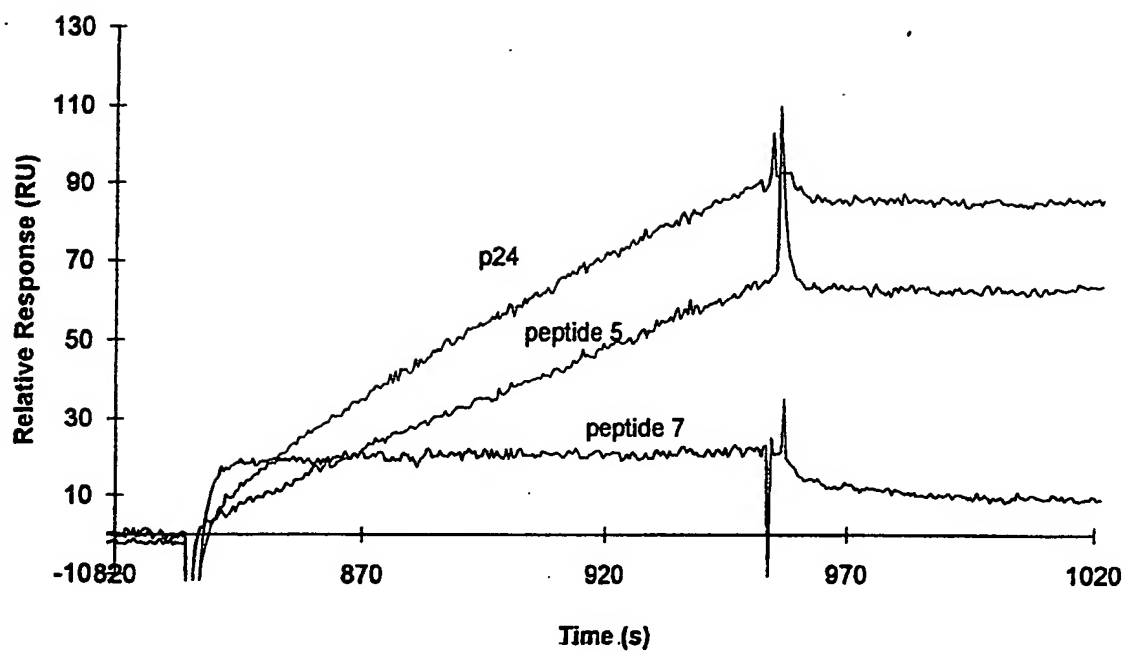FIG. 4
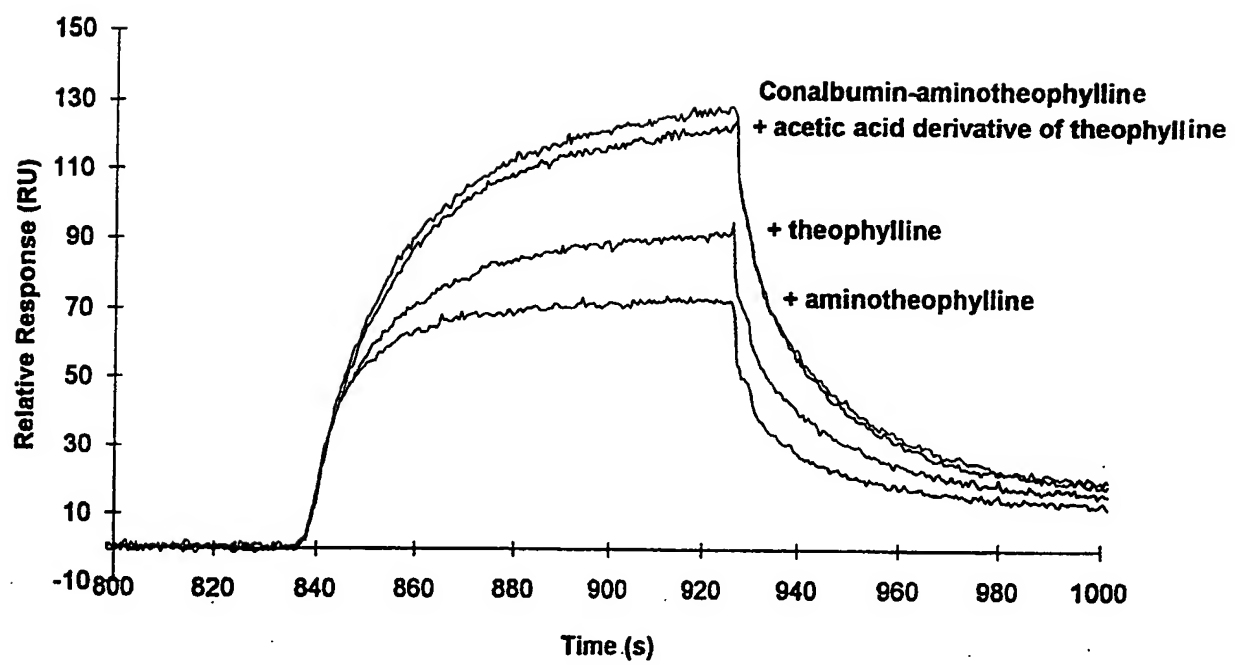
FIG. 5

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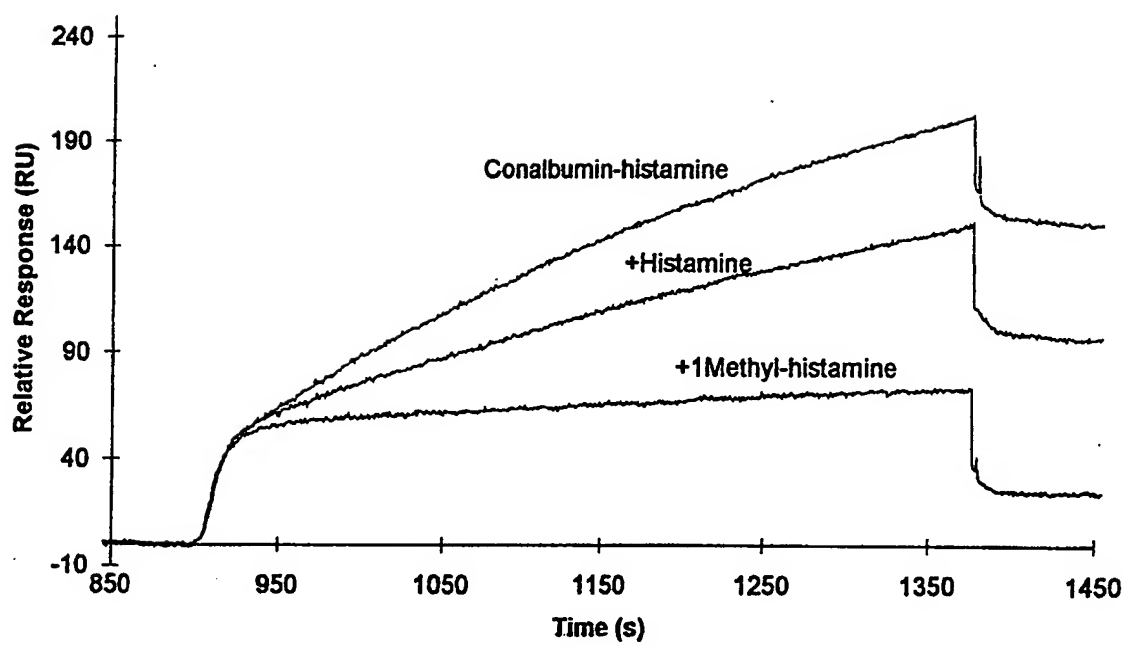
FIG.6

INTERNATIONAL SEARCH REPORT

	International application No.
	PCT/SE 94/00480

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5: G01N 33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE BIOSIS SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biosensors & Bioelectronics, Volume 6, 1991, Kim R. Rogers et al, "Pharmacological specificity of a nicotinic acetylcholine receptor optical sensor", page 507 - page 516, see page 511-512	1-11
	--	
Y	WO, A1, 9005305 (PHARMACIA AB), 17 May 1990 (17.05.90), see pages 2, 17-19 and 22 and figures 7a - 8b	1-11
	--	
Y	WO, A1, 9005306 (PHARMACIA AB), 17 May 1990 (17.05.90), see pages 7-8 and claims	1-11
	--	

☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document but published on or after the international filing date	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 1994	02 -09- 1994
Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9005305	17/05/90	EP-A-	0442922	28/08/91
WO-A1- 9005306	17/05/90	EP-A-	0442922	28/08/91
		EP-A-	0442930	28/08/91